Genetic diversity of *Amylostereum areolatum*, the fungal symbiont of the invasive woodwasp *Sirex noctilio* in South Africa

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Abstract

Amylostereum areolatum is a wood-decaying homobasidiomycete fungal symbiont of *Sirex noctilio*. Together they cause serious damage to pine plantations in the Southern Hemisphere. The fungus reproduces asexually and is vertically transmitted by *S. noctilio* females, which results in extensive spread of clones. Specific *A. areolatum* clones are often dominant in areas invaded by the wasp. This is in contrast with the high diversity and complex invasion pattern of *S. noctilio* in most of these regions. In South Africa, for example, nuclear and mitochondrial ribosomal loci and vegetative compatibility groups (VCGs) markers on a small number of isolates have shown that only one genotype is present in the country. The aim of this study was to develop microsatellite markers for *A. areolatum* and determine the genetic diversity and structure of a relatively large collection of isolates of the fungus in South Africa. From five sequenced *A. areolatum* genomes, a total of 233 microsatellite primer pairs were designed, of which 57 were polymorphic amongst the genomes. Eleven of these polymorphic markers were then used in a population genetics study including 55 South African isolates. Surprisingly, nine multilocus genotypes were found amongst these isolates, and with no population structure amongst different regions across South Africa. The single VCG previously identified for isolates from the country, clearly do not correspond to a clone. The detected *A. areolatum* variation has relevance for the biology of the *Sirex-Amylostereum* symbiosis, its introduction history into South Africa and its management through biocontrol. The microsatellite primers and data emerging from them also provide powerful tools for the study of *A. areolatum* populations in other parts of the world.

KEYWORDS: *Amylostereum areolatum*, population genetics, microsatellites, genome mining, sirex woodwasp, biological control

1. Introduction

Amylostereum areolatum (Chaillet: Fries) Biodin is a white-rot wood-decaying homobasidiomycete fungus that is a symbiont of Siricid woodwasps (Siricidae; Hymenoptera). This fungus is associated with *Sirex noctilio* Fabricius, an invasive woodwasp that infests *Pinus* spp. in many parts of the world (Slippers, Hurley, & Wingfield, 2015). Asexually produced arthrospores are carried by female *S. noctilio* in internal mycangia situated at the base of the female ovipositor (Coutts & Dolezal, 1969; Slippers, Coutinho, Wingfield, & Wingfield, 2003). *Sirex noctilio* inoculates its eggs with *A. areolatum* arthrospores, and oviposits along with a phytotoxic venom into the woody stems of trees. The joint action of the venom and fungus from multiple attacks can result in tree death (Bordeaux et al., 2014; Coutts, 1969; Spradbery, 1973). Degradation of cellulose and lignin by *A. areolatum* is essential for nutrient acquisition of *S. noctilio* larvae (Adams et al., 2011; Talbot, 1977; Thompson, Bodart, McEwen, & Gruner, 2014). Before the female adult *S. noctilio* emerge, they acquire the asexually produced arthrospores into their mycangia (Talbot, 1977).

The asexual reproduction of *A. areolatum* and vertical transmission between generations by *S. noctilio* result in extensive spread of clones of the fungus (Slippers et al., 2015). Widespread clones of *A. areolatum*, defined as vegetative compatibility groups (VCGs), were first reported in Scandinavia (Thomsen & Koch, 1999; Vasiliauskas & Stenlid, 1999; Vasiliauskas, Stenlid, & Thomsen, 1998). For example, Vasiliauskas et al. (1998) reported an *A. areolatum* clone spread across 1000 km area, which included Sweden, Denmark and Lithuania. Another dominant clone in that study occurred in both Sweden and Lithuania. These two clones constituted 34 of the 53 isolates obtained from the area (Vasiliauskas et al., 1998). In total, Vasiliauskas et al. (1998) found 12 *A. areolatum* clones with low genetic differentiation among them. More recently, Fitza et al. (2016) showed that populations of *A. areolatum* and *A. chailletii* associated with Japanese siricid species also had high degree of clonality.

These studies support the hypothesis that vertical transmission of *Amylostereum* spp. by Siricids is the prevalent mode of distribution, although horizontal transmission is also possible (Slippers et al., 2015).

In its invasive range in the Southern Hemisphere, *A. areolatum* populations have very low diversity. Slippers, Wingfield, Coutinho, & Wingfield (2001) found only two VCGs for isolates from South Africa, South America and Australasia. These results suggest a limited introduction into the region, resulting in a genetic bottleneck, predominantly asexual reproduction and transmission by the wasp, including movement between these continents probably by anthropogenic pathways. In contrast, Boissin et al. (2012) showed that the *S. noctilio* invasion across the Southern Hemisphere was more complex and included multiple introductions from multiple source populations. Most of the invasions appeared to be admixed populations from independent introductions originating in the native ranges as well as from the invaded areas. These contrasting results could be due to the inability of VCGs to reveal population diversity in *A. areolatum*, the limited geographical sampling in earlier studies, the acquisition of dominant local fungal genotypes by newly introduced wasp genotypes or introgression of newly introduced variation into resident wasp populations independent of fungal introductions (Slippers et al., 2015).

Amylostereum areolatum is not only essential for the growth and development of *S. noctilio*, but also for one of the wasps natural enemies; the parasitic nematode *Deladenus siricidicola* (Bedding, 1972; Zondag, 1969). This nematode feeds and can be mass-reared on *A. areolatum* during one phase of its life cycle, but infects and sterilizes the female wasps during another phase (Bedding & Iede, 2005). The nematode is the main biological control agent used in the management of *S. noctilio* across Australasia, South America and South Africa (Hurley, Slippers, & Wingfield, 2007). It is known that diversity in the fungus can dramatically influence the productivity of the nematode (Morris, Jimenez, Long, Williams, & Hajek, 2012). For example, when the nematode was grown on different strains of the fungus, Morris et

al. (2012) found up to 100-fold difference in the number of offspring after 25 days. Understanding the diversity of *A. areolatum* in its invaded range is thus important, not only to understand the biology and invasion history of the woodwasp, but also in order to develop effective biological control strategies using *D. siricidicola*.

The diversity of *A. areolatum* has been studied using only sequence data of nuclear and mitochondrial genic regions and VCGs (Bergeron et al., 2011; Castrillo et al., 2015; Hajek, Nielsen, Kepler, Long, & Castrillo, 2013; Slippers et al., 2001; Thomsen & Koch, 1999; Vasiliauskas & Stenlid, 1999; Vasiliauskas et al., 1998). The DNA markers are relatively conserved within the species, while VCGs can be difficult to interpret and do not show a directly proportional relationship with genetic distance. The use of more variable and co-dominant markers, such as microsatellites, would add to our understanding of *A. areolatum* diversity, *Sirex-Amylostereum* symbiosis and this could improve the management of the wasp using *D. siricidicola.* The aim of this study was consequently to develop microsatellite markers for *A. areolatum.* These markers were then used to determine the genetic diversity and structure of a collection of *A. areolatum* isolates from South Africa. We also compared the utility of microsatellites and VCGs as markers to assess the genetic variation of *A. areolatum* in South Africa.

2. Materials and Methods

2.1. Fungal isolates and DNA extraction

Total genomic DNA was extracted from five *A. areolatum* strains originating from Argentina, Australia, South Africa and New Zealand, collected between 1962 and 2010 (Table 1) following the approach described by Slippers et al. (2001). The total genomic DNA of these strains was sequenced using HiSeq

Illumina platform (Fasteris, Switzerland) where the paired end libraries had a 100 bp read length with a 300 bp insert size.

Table 1. *Amylostereum areolatum* strains used for genome sequencing.

Country	Year isolated	Isolate reference
Argentina	2005	Arg8
Australia	2001	CMW6863
South Africa	2011	RSA_NK1
New Zealand	1962	CMW37110
New Zealand	2010	NZ_K17A

For the population genetic study, a total of 57 *A. areolatum* isolates were collected in South Africa, Australia and United States of America (Table 2). Of these, 55 South African isolates were the main focus of the analyses, the two remaining isolates were included for comparative purposes. The South African isolates were obtained from female wasps from four pine-growing regions of South Africa i.e. the Eastern Cape, KwaZulu-Natal, Mpumalanga and Western Cape provinces. All *A. areolatum* isolates were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. These isolates were grown on MEA (30 g l^{-1} agar, 20 g l^{-1} malt extract) for 7 days at room temperature before genomic DNA was extracted using the technique described by Duong, de Beer, Wingfield, & Wingfield (2012).

Table 2. *Amylostereum areolatum* isolates used for microsatellite analysis.

Region	Year isolated	Locality	Isolate number
Eastern Cape	2002	NECF plantation	CMW11315
	2003	NECF plantation	CMW13653
		Langeni plantation	CMW13836
		NECF plantation	CMW13838
	2014	Hogsback	CMW43964
		Hogsback	CMW43965
		Hogsback	CMW43966
		Hogsback	CMW43967
	2015	Hogsback	CMW44292
		Quar(Geoparks)	CMW44293
		Quar(Geoparks)	CMW44294
KwaZulu-Natal	2003	Kubusie plantation	CMW13829
		Goodhope plantation	CMW13832
		Sutton plantation	CMW13835
		Linwood plantation	CMW13656
		Blairothol plantation	CMW13657
		Epson plantation	CMW13658
	2007	Unspecified	CMW29657
	2006	NCT plantation	CMW29659
	2013	RSA14	CMW46043
		RSA33	CMW47563
	2014	Epsom	CMW43945
		Underberg	CMW43946
		Pinewoods	CMW43947
		Pinewoods	CMW43948
		Holmesdale	CMW43949
		Wildebeest	CMW43950
		Funeray	CMW43951
		Funeray	CMW43952
Mpumalanga	2002	Singers/Umtata	CMW10521
	2014	Lothair	CMW43953
		Helo	CMW43954
		Woodstock	CMW43955
		Woodstock	CMW43956
		Rooihoogte	CMW43957
		Rooihoogte	CMW43958
		Torburniea	CMW43959
		Sjonajona	CMW43960

		Highlands	CMW43961
		Jessievale	CMW43962
Western Cape	1997	Unknown	CMW8900
	2002	Pine Grove	CMW9745
		Kluitjieskraal plantation	CMW9894
		Papagoaiberg	CMW10300
	2003	Riversdale	CMW11582
		Bergplaas plantation	CMW11406
		Garcia plantation	CMW11411
		Kluitjieskraal plantation	CMW11413
		Kouma plantation	CMW11414
	2007	Wolwekloof	CMW29663
	2013	Witelsbos	CMW40875
	2015	Ruigtevlei	CMW44285
		Bracken	CMW44286
		Buffelsnek	CMW44290
		Geelhoutvlei	CMW44291
Australia			CMW40871
USA 13028	2014	Delmar	CMW40703

2.2. Microsatellite genome mining and polymorphic marker identification

Microsatellite containing contigs were identified using MSATfinder (Thurston & Field, 2005) from whole genome assemblies for each of the five *A. areolatum* strains (Table 1), ranging from dinucleotide to hexanucleotide repeat motifs. Microsatellite motifs flanked by 100 bp nucleotides at either side were separated into repeat motif-defined files using a phython script extractor. The New Zealand NZ_K17A strain was used as a reference to design primers using the online program PRIMER3 (http://primer3.ut.ee/) (Rozen & Skaletsky, 2000; Untergasser et al., 2012).

Developed primer sequences were used to screen microsatellite defined files in isolates from Argentina (Arg8), Australia (CMW6863), South Africa (RSA_NK1) and New Zealand (CMW37110) to identify polymorphic microsatellite markers *in silico*. Twenty-eight polymorphic markers (Figure1) present across

all *A. areolatum* strains were selected based on sequence differences and estimated observable fragment size variation using gel electrophoresis. The 28 polymorphic markers included 16 trinucleotides, and six dinucleotides and tetranucleotides, respectively. These markers were tested on five *A. areolatum* isolates (three South African – CMW43948, CMW43962, CMW8900, and two Canadian – CMW37037 and CMW37019). PCR was performed in a 25 µL reaction volume using a ProFlexPCR System (Applied Biosystems, USA).

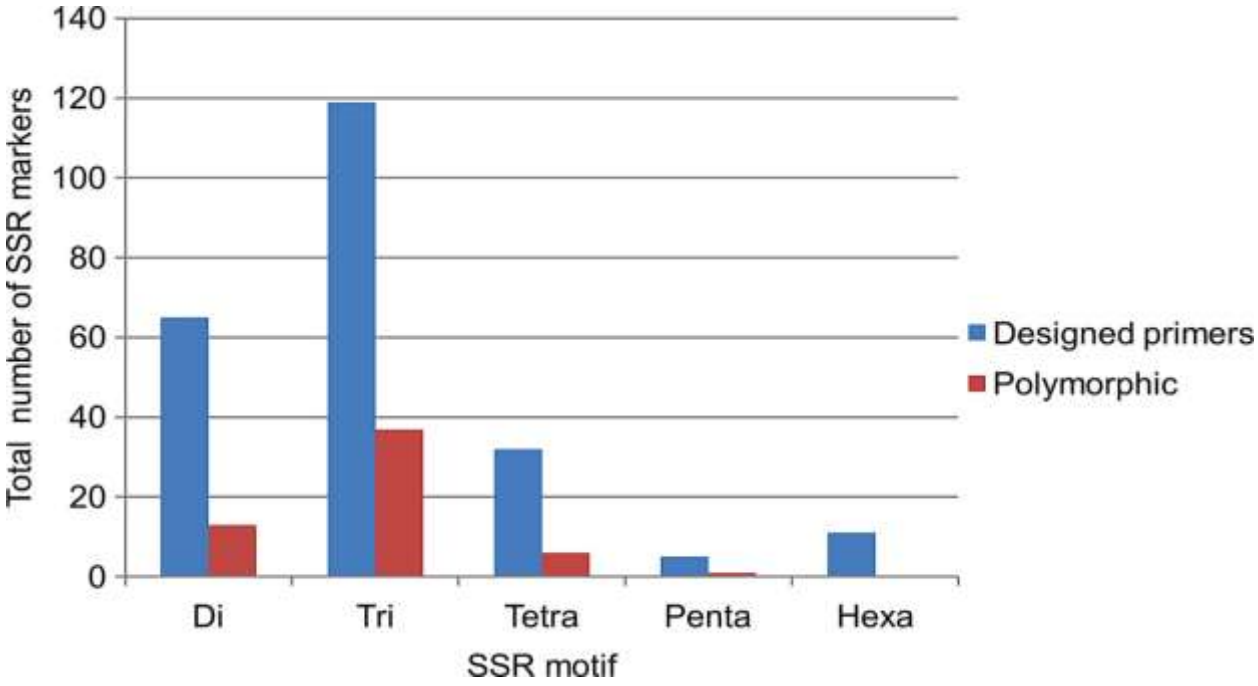

Figure 1. Microsatellites mined from *Amylostereum areolatum* isolate NZ_K17A.

The PCR reaction mix included 4 µl of 10 mM dNTPs, 2.5 µl of 10X PCR buffer (10X solution, 100 mM Tris-HCl, 500 mM KCl, pH 8.3) (Roche Diagnostics GmbH, Germany), 2 µl of 25 mM $MgCl_2$ (Roche Diagnostics GmbH, Germany), 1 µlof 10 µM of each forward and reverse primer, 0.5 µl of FastStart *Taq* DNA polymerase and 12 µl of sterilized SABAX water to make up the total volume of 25 µl. The PCR conditions were an initial denaturation step at 95 °C for 4 minutes followed by 35 cycles of denaturation

at 94 °C for 1 minute, annealing at 57 °C or 58 °C for 30 seconds, extension at 72 °C for 1 minute; with a final extension at 72 °C for 10 minutes. Fragment size variation of PCR products was observed after electrophoresis on a 2% gel agarose gel stained with GelRed™ (Biotium Incorporation, USA) while visualized on a Gel Doc™ EZ Imager (Bio-Rad, USA). Estimation of PCR product size was done using a 100 bp molecular weight marker (Fermentas, USA). From these results, microsatellite markers were selected based on observed fragment variations between isolates, and subsequently fluorescently labelled for application in population genetics studies (Table 3).

2.3. Microsatellite genotyping

The PCR volume used and product visualization was performed as mentioned above, while the PCR conditions included an initial denaturation step at 95 °C for 4 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C or 58 °C for 30 s, extension at 72 °C for 1 min; and a final extension at 72 °C for 45 min. Two panels containing seven and four microsatellites markers (Table 3) respectively, per strain were used. The PCR amplicons in a solution containing 14:1000 ratio of Liz-500 size standard and HiDi™ Formamide were analysed on a 3500xl Genetic Analyzer (Applied Biosystems, USA). Allele sizes were scored using GeneMapper® Software version 5.0 (Applied Biosystems, USA).

2.4. Genetic diversity

The 57 *A. areolatum* isolates were grouped according to their geographic origins. Characterisation of microsatellite markers included all isolates and was performed using the program POPGENE version 1.3 (Yeh & Boyle, 1997). Allelic evenness was determined using the statistical package R (R Core Team, 2013) and the programme Poppr (http://cran.r-project.org/package=poppr) (Kamvar, Tabima, & Grünwald, 2014). Private alleles were calculated using GenAlEx version 6.5 (Peakall & Smouse, 2006). Summary statistics for *A. areolatum* in pine growing areas of South Africa included calculations for expected multilocus genotypes (MLG) based on a rarefraction method, to account for population size

Table 3. Eleven polymorphic microsatellite markers used in *Amylostereum areolatum* population genetics study.

Locus	Primer name	Primer sequences (5' to 3')	Repeats range	Tm (°C)	Fluorescent label	Panel	Expected size (bp)[a]
Aa3-4	Aa3-4F	GTCCAGGCTATCGTGTGAGA	$(CCT)_{8\text{-}\mathbf{12}}$	58.9	VIC	1	229
	Aa3-4R	CCCGTTCCCTCTCAAGCTAA		59.1			
Aa3-5	Aa3-5F	GTGAGACAAAGCGTGGTTGG	$(AGG)_{8\text{-}\mathbf{9}}$	59.69	NED	1	200
	Aa3-5R	GATCTCCGTCTCGTCCACTC		59.34			
Aa3-7	Aa3-7F	TGAATGGATGGCACGAGAGA	$(AGG)_{8\text{-}\mathbf{11}}$	58.81	PET	1	230
	Aa3-7R	GACATCGTTCCCCTTCCCTT		59.38			
Aa3-8	Aa3-8F	AGAGGCATTTTCAGCGTCTG	$(AGG)_{\mathbf{10}\text{-}12}$	58.55	FAM	1	189
	Aa3-8R	GACGTGCAGAGTTCATCCAC		58.93			
Aa3-9	Aa3-9F	TGCGATTGTGAGGTTTGTGG	$(GCT)_{\mathbf{11}\text{-}12}$	59.05	NED	1	246
	Aa3-9R	GCAGGCCCCATCAAATCAAA		59.1			
Aa3-14	Aa3-14F	TCATCGACCAAAGCCAGACT	$(ACG)_{\mathbf{8}\text{-}9}$	59.02	PET	2	223
	Aa3-14R	GAATGCTGTTGACGCCTGAT		58.91			
Aa3-23	Aa3-23F	GTTTAGGACTGCACGTGGGA	$(CCG)_{\mathbf{8}\text{-}12}$	59.97	FAM	1	246
	Aa3-23R	CTCGACCCTCAAGAACGTGT		59.69			
Aa3-24	Aa3-24F	TACGACTCCCTGTTCAACGG	$(ACG)_{\mathbf{12}\text{-}15}$	59.4	NED	2	226
	Aa3-24R	GCGAAACGTCATACAGAGCT		58.37			
Aa3-25	Aa3-25F	CGAATGGGATGTCAGCGTG	$(ACG)_{8\text{-}\mathbf{12}}$	58.99	FAM	2	237
	Aa3-25R	ATCACCATCACCTCATCGCT		58.87			
Aa4-3	Aa4-3F	GAAGACGACGTGAGGAGGAG	$(ATGT)_{\mathbf{6}\text{-}10}$	59.55	VIC	2	206
	Aa4-3R	CTTTCTTCCACCGCCTACTG		58.27			
Aa4-5	Aa4-5F	TGAAGGGCGATGATTCCACT	$(TTTC)_{7\text{-}10}$	59.09	PET	1	178
	Aa4-5R	TTTGGCCCCTTTGTTTCTCG		58.96			

[a] based on New Zealand NZ_K17A strain

differences. It also contained four different measures of genotypic diversity, namely Shannon-Weiner index (Shannon, 2001), Stoddart and Taylor's index (Stoddart & Taylor, 1988), Simpson's index (E. Simpson, 1949) and Nei's unbiased genotypic diversity (Nei, 1978). These statistical measures and the multilocus genotype evenness were calculated using R package (R Core Team, 2013) Poppr (Kamvar et al., 2014) with mean number alleles determined using POPGENE version 1.31 (Yeh & Boyle, 1997).

2.5. Population structure

Principal Component Analysis (PCoA) was performed, using the co-dominant genetic distance matrix and standardized distance PCoA method, in GenAlEx version 6.5 (Peakall & Smouse, 2006) to determine population structure. Analysis of molecular variance was conducted in GenAlEx version 6.5 (Peakall & Smouse, 2006) using 999 permutations to test for partitioning of molecular variance within and between populations. Estimated gene flow, calculated based on Nei's unbiased genetic distance, and genetic differentiation (F_{st}) values among the different populations in South Africa was determined using GenAlEx version 6.5 (Peakall & Smouse, 2006).

2.6. Vegetative Compatibility

To determine vegetative compatibility groups (VCGs) for South African *A. areolatum* isolates, two isolates per MLG were selected (unless a MLG included only one isolate) and cultured. From the growing fungal cultures, plugs were inoculated at the centres of Petri dishes containing the MEA (30 g l^{-1} agar, 20 g l^{-1} malt extract) with the fungal surface facing downwards. Each inoculated MEA plate contained two plugs, 1 cm apart, for heterokaryon pairing. Each plug represented a MLG. All 105 possible combinations of pairing were performed. Plates were wrapped with parafilm. The incubation period and compatibility scoring method was conducted using the technique described by Slippers et al. (2001).

3. Results

3.1. Microsatellite genome mining and polymorphic marker identification

Assembled contigs for the five *A. areolatum* strains ranged from 12 101 to 19 871 in number, of which between 377 to 439 contained microsatellite repeats (Table S1). Microsatellite motifs varied from dinucleotides to hexanucleotides, with the most abundant being trinucleotides while pentanucleotides were the least abundant (Table S1). From the reference strain, a total of 233 microsatellite primers were designed (data not shown) and represented the microsatellite motifs mentioned earlier (Table S2). Of these potential markers, 57 were found to be polymorphic by comparing alleles between the sequenced strains (Figure 1). Twenty eight of these were selected based on the variation in allele size. These amplicons were visualised using electrophoresis on a 2% agarose gel and based on the observable fragment size variation, 11 were selected to be fluorescently labelled and used in population genetics studies (Table 3).

3.2. Microsatellite genotyping

The 11 selected microsatellite primers amplified between two and five alleles revealing a total of 27 alleles across all populations (Table 4). Four loci had unique alleles in Australian, South African and the USA isolates (Table S3). The Australian isolate (CMW40871) showed the greatest number of unique alleles ($N_{private}$ = 3) (Table S3). Eleven multilocus genotypes were detected in this population genetics study (Table S4, S5).

Table 4. Microsatellite characterisation of *Amylostereum areolatum* isolates used in the study.

Locus	N_a	Allelic range	H_o	H_e	F_{is}	Allelic evenness
Aa3-4	2	219-226	0.0702	0.3706	0.8106	0.789
Aa3-5	2	200-210	1	0.5	-1	1
Aa3-7	3	218-230	0.9643	0.5386	-0.8065	0.897
Aa3-8	2	189-195	1	0.5	-1	1
Aa3-9	2	248-251	1	0.5	-1	1
Aa3-14	2	222-225	1	0.5	-1	1
Aa3-23	2	244-253	0	0.0345	1	0.387
Aa3-24	5	204-234	1	0.627	-0.595	0.836
Aa3-25	2	222-237	1	0.5	-1	1
Aa4-3	2	206-222	0.0351	0.0997	0.6481	0.484
Aa4-5	3	174-186	1	0.5086	-0.9661	0.949

Notes. F_{is}: inbreeding coefficient; H_e: expected heterozygosity; H_o: observed heterozygosity; N_a: number of alleles.

Nei, 1978 unbiased gene diversity estimate.

3.3. Genetic diversity

Allele distribution was even for five markers, near even for four and uneven for two markers in the dataset (Table 4).The mean number of alleles was close to two for all regions in South Africa (Table 5). From the eleven multilocus genotypes (MLG) found; the two isolates from Australia and the USA each had a unique MLG (Table S4), while the 55 South African isolates had nine MLGs. Of these, five MLGs were found in the Western Cape (WC), four in KwaZulu-Natal (KZN) and three each in the Eastern Cape (EC) and Mpumalanga (MP) (Table 5). KwaZulu-Natal, EC and MP isolates shared two MLGs; KZN and MP isolates shared one MLG, EC and WC shared one; and four and one unique MLGs were found in WC and KZN, respectively (Figure 2; Table S4). The abundance of MLGs in EC and MP was similar while KZN and WC showed a predominance of MLG 6 and MLG 7, respectively (Figure 2). The level of heterozygosity was similar and intermediate among the regions in South Africa, ranging from 0.395 to 0.433 (Table 5).

Table 5. Summary statistics of *Amylostereum areolatum* in South Africa.

Population	N	Mean A	MLG_T	eMLG (± SE)	H	G	λ	E.5	He
Western Cape	15	2	5	3.9 (0.774)	1.081	2.1	0.524	0.567	0.426
KwaZulu-Natal	18	1.9091	4	2.93 (0.751)	0.761	1.6	0.377	0.53	0.398
Eastern Cape	12	1.9091	3	2.98 (0.122)	1.011	2.57	0.611	0.898	0.433
Mpumalanga	10	1.8182	3	3	1.055	2.78	0.64	0.95	0.431
Total	55		9	9	1.64	4	0.75	0.72	0.422

Note. λ: Simpson's index (Simpson, 1949); ± SE: standard error based on eMLG; A: number of alleles; E.5: MLG evenness; eMLG: rarefaction number of expected multilocus genotypes based on the smallest population size; G: Stoddart and Taylor's index of MLG diversity (Stoddart & Taylor, 1988); H: Shannon–Wiener index of MLG diversity (Shannon, 2001); MLG_T: total number of multilocus genotypes; *N*: number of isolates.

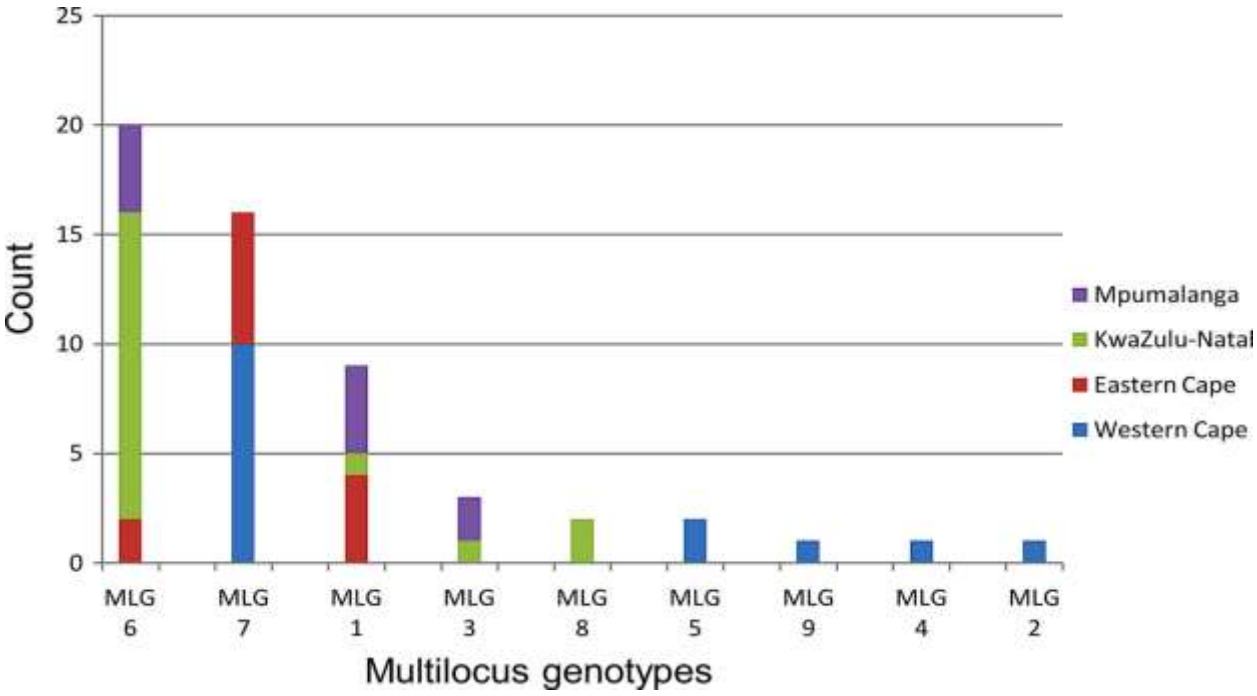

Figure 2. South African *Amylostereum areolatum* multilocus genotype diversity in South Africa.

3.4. Population structure

There was no population structure inferred by PCoA analysis (Figure 3). Analysis of molecular variance (AMOVA) showed that the majority of the variation was within populations rather than between populations (Figure S1). The estimated gene flow between the regions in South Africa ranged from 5.008

to 27.458. The genetic differentiation (F_{st}) between regions showed WC and MP were the most differentiated, while EC and MP were the least. Eastern Cape was least differentiated from KZN than WC (Table 6).

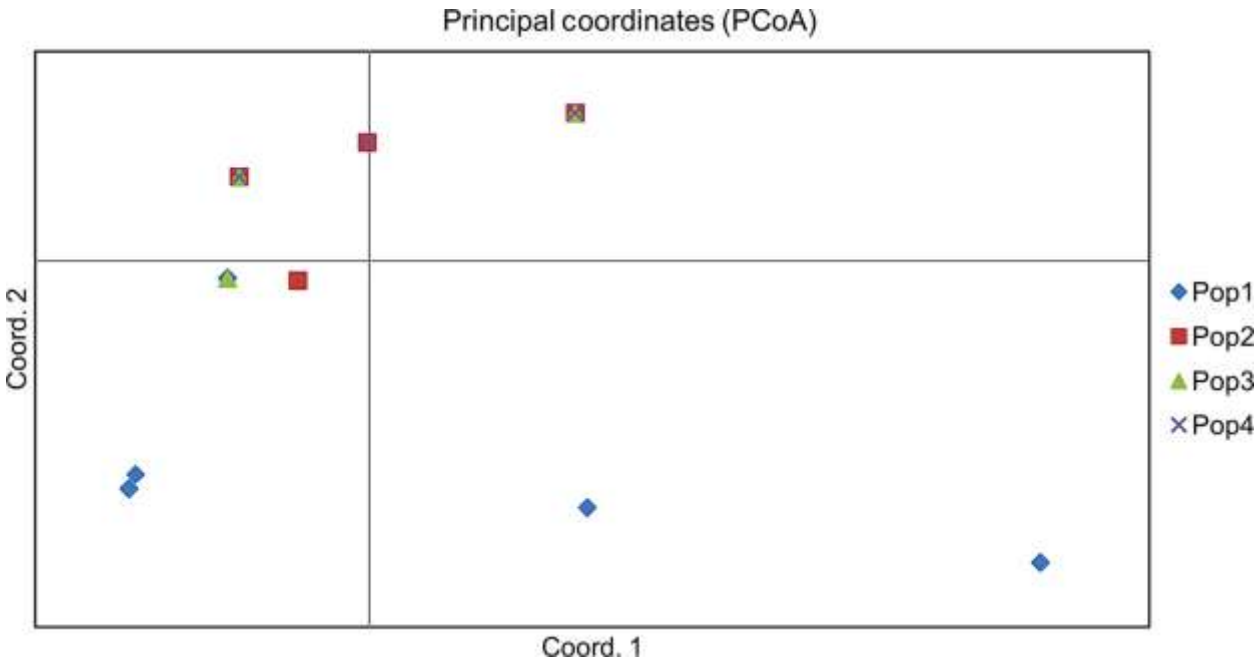

Figure 3. PCoA of *Amylostereum areolatum* isolates from South Africa showing no population structure. Pop1 refers to isolates in the Western Cape, Pop2 refers to isolates in KwaZulu-Natal , Pop3 refers to isolates in the Eastern Cape and Pop4 refers to isolates in Mpumalanga.

Table 6. Pairwise comparison of estimated gene flow (N_m) (below diagonal) and genetic differentiation (F_{st}) (above diagonal) among South African isolates of *Amylostereum areolatum*.

	Western Cape	Eastern Cape	KwaZulu-Natal	Mpumalanga
Western Cape	-	0.022	0.023	0.048
Eastern Cape	11.227	-	0.018	0.009
KwaZulu-Natal	10.522	13.959	-	0.024
Mpumalanga	5.008	27.458	10.225	-

3.5. Vegetative Compatibility

All South African isolates were somatically compatible, irrespective of the MLG to which they belonged (Table S6). Growth rate among isolates differed although mycelia intermingled freely. Interaction between isolates and colony morphologies were not uniform compared to controls.

4. Discussion

Eleven microsatellite (SSR) markers were developed and applied in this study to characterise the genetic diversity of *A. areolatum* isolates from different parts of South Africa. Results revealed significantly greater diversity than previously reported (Slippers et al., 2001) including nine multilocus genotypes. No population structure was found among the *A. areolatum* isolates that were from a wide diversity of sites in South Africa. The diversity revealed by the SSR markers was not reflected by the diversity of VCGs in these populations. This result illustrates the higher resolution provided by microsatellite markers in contrast to VCGs.

We mined 233 microsatellite markers from the whole genome sequences of *A. areolatum* isolates, of which eleven were ultimately used in this study. Traditionally development of microsatellite markers has been laborious and expensive (Burgess, Wingfield, & Wingfield, 2001; Cortinas, Barnes, Wingfield, & Wingfield, 2006; Santana et al., 2009; Slippers et al., 2004). The advent of genome sequencing has enabled genome-wide screening for microsatellites (Cai, Leadbetter, Muehlbauer, molnar, & Hillman, 2013; Karaoglu, Lee, & Meyer, 2005; Li et al., 2009; Lim, Notley-McRobb, Lim, & Carter, 2004; Moges et al., 2016; M. C. Simpson, Wilken, Coetzee, Wingfield, & Wingfield, 2013) and with continuous reduction in genome sequencing costs (Muir et al., 2016) a powerful and cost effective tool for microsatellite discovery has emerged.

The high level of MLG diversity for South African isolates of *A. areolatum* in this study was surprising. Previous studies (Slippers, Wingfield, Coutinho, & Wingfield, 2002; Slippers et al., 2001) have shown that the South Africa population of this fungus is represented by only a single VCG. This was interpreted as representing a single clone, suggesting a single or limited introduction together with *S. noctilio* (Slippers et al., 2002; Slippers et al., 2001). Subsequent to those studies, Boissin et al. (2012) suggested there had been multiple independent introductions of *S. noctilio* into South Africa. Results of the present study support those of Boissin et al. (2012) showing a considerably greater level of diversity than originally recognised.

The genetic diversity detected in the *A. areolatum* populations in South Africa displayed no geographic population structure. The majority of the variation occurred within rather than between the regions. These results suggest a single population of *A. areolatum* in South Africa. This likely reflects movement of the wasp and fungus between regions. In the Eastern parts of the country where there are relatively continuous plantations of *Pinus*, much of the connection between populations would most likely have been via natural spread. In this regard, the wasp is known to fly up to 50km per year (Bruzzone, Villacide, Bernstein, & Corley, 2009), which would enable effective local spread.

The most unique diversity in *A. areolatum* occurred in the Western Cape, where the wasp has been present for the longest period of time (Tribe, 1995). But there were also unique MLGs in the eastern parts of the country (Eastern Cape, Kwazulu-Natal and Mpumalanga) that did not occur in the Western Cape. Thus, while all regions share some diversity and this has likely been influenced by a single source population, the possibility that independent introductions into the Eastern and Western populations in South Africa occurred cannot be ruled out (Boissin et al., 2012).

Vegetative compatibility tests conducted in this study revealed the presence of a single VCG, which is similar to that found previously by Slippers et al. (2001). Application of our microsatellite markers

provided clear evidence that this VCG does not represent a single clone. This is not surprising because a VCG tests on agar are suggested to underestimate diversity (van der Nest et al., 2012) . Vasiliauskas et al. (1998) confirmed that the VCGs in Scandinavia were clones by genetic profiles determined by minisatellites. However, minisatellites are not locus-specific and could underestimate diversity in VCGs (Schlötterer, 2004). For this reason, it would be worth studying the clonality of European or Japanese *A. areolatum* populations using the microsatellites developed here.

Variation in populations of *A. areolatum* found in this study could impact on the Sirex biological control program in South Africa. Mass production of *D. siricidicola* depends on *A. areolatum*, as does the reproduction of the nematode within trees (Nahrung, Ramsden, Hayes, Francis, & Griffiths, 2016). The strain of the fungus, however, has a major influence on the productivity of *D. siricidicola* (Morris et al., 2012). In North America *D. siricidicola* showed differential productivity among the five *A. areolatum* strains present (Morris et al., 2012). Another recent study showed more than a 300 fold difference in populations of the nematode after 25 days culturing on different strains of *A. areolatum* (Mlonyeni, Wingfield, Greeff, Wingfield, & Slippers, unpublished). Although, mechanism underlying the differential nematode productivity on *A. areolatum* strains remains to be understood, a possible factor may be a reverse ability of *A. areolatum* to parasitize *D. siricidicola* (Morris & Hajek, 2014).

It is clear, that the South Africa biological control program should consider the productivity capacity of *D. siricidicola* on the various *A. areolatum* strains. This is especially relevant in light of the fact that the nematode used in the biological control program lacks genetic diversity (Mlonyeni et al., 2011). Knowledge arising from the present study should facilitate such studies, not only by identifying the diversity of genotypes of *A. areolatum* in the country, but also by providing and identify for the dominant genotypes in different regions. The latter information would be valuable for nematode rearing programs aimed to introduce the nematode into new environments.

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